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# **How to Answer the Question — Are Drugs Real Threats to Biological Systems or Overrated Innocuous Chemicals?**

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Bruno Nunes

Additional information is available at the end of the chapter

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## **1. Introduction**

Recent years have brought a new awareness about the potential deleterious environmental impacts of a multiplicity of anthropogenic substances. We have now reached a scientific/technological standpoint that allows the prediction of most likely effects posed by large groups of substances, including pharmaceutical drugs, and their effects on exposed biota. However, as is the case of pharmaceutical drugs, scarce are the studies and unequivocal data that establish a direct linkage between their environmental presence and dispersal, and toxicity in exposed organisms. Several drawbacks are systematically invoked by detractors of the issue of pharmaceutical contamination when considering this issue, from the unmistakable low levels in which drug residues are found, to the absence of effects caused by metabolites being excreted from biologic systems. However, one cannot discard the evidences: drug residues are present in most environmental matrices, including the particular case of the aquatic compartment; the number of drugs, their metabolites and degradation products detected in these environmental matrices is alarmingly high, and never stopped increasing since their first detections; some of these drugs are not characterized in terms of toxicity towards the majority of exposed organisms, and their toxic outcomes are unpredictable; the use, release and presence of these substances will not end, or be decreased in a near future, a factor that should, at least, work as an additional stimulus for the development of research into this field.

Therapeutic agents, both human and veterinary, are modern commodities that make part of the developed society. These chemicals are usually developed to fulfil a series of criteria, mainly effectiveness, safety, comfort of use, therapeutic success, and low incidence of side effects. However, the issue of environmental fate of these molecules has only recently been raised, and the main approach established in international guidelines (e.g. European Medi-

cines Agency, EMA) takes only in consideration the expected levels in which these compounds may occur in the aquatic compartment. Thus, a consensual precautionary principle was adopted, considering only the levels of dispersion, based on estimates of consumption, that drugs may undergone. No specific guidelines or testing protocols were ever developed to analyse the biological effects of pharmaceuticals in the environment, and efforts devoted so far to this problematic have always relied on the quest, validation and proposal of more accurate and reliable analytical methodologies. However, rather than quantifying the presence or levels of drugs in the environment, an integrative approach that characterizes their toxic effects on multiple components of the ecosystem is now much required. Only with results from a comprehensive, well-suited battery of multi-species biotests, complemented by a systematic survey of published data, it will be possible to answer to the big question: are drugs in the environment real threats to biological systems, or overrated innocuous chemicals?

The main drawback of studying the potential deleterious effects of drugs in the environment is the type of compound that one might expect to find. Considering that a rough estimate of the number of distinct substances presently in use in Europe is around 3000, it is possible to sustain the complexity of this task. Taking into consideration that a considerable number of these substances share a similar pharmacology/toxicology, we can reduce this number surely to a certain hundreds; however, being metabolised and excreted, the formation of metabolites and degradation products will increase again the number of substances that one can find in the wild. An additional factor to consider is the absence of toxicity data for the majority of metabolites and degradation products. For all the given reasons, the ecotoxicological profile of most therapeutic drugs is largely unknown, and a large effort must be devoted to the proposal, validation and use of a comprehensive set of biomarker tools. This effort will be mandatory to diagnose exposure to pharmaceuticals for a vast number of species, and to predict the magnitude of the threat posed by pharmaceutical compounds to non-target organisms.

Even if this approach is satisfactorily followed, the discrepancy of experimental data can be another factor to consider. For some drugs, a considerable amount of scientific data is now available, which should facilitate the interpretation of their ecotoxicological profiles and risks. However, this is not always a simple and immediate task to perform. In fact, for some drugs, the already compiled information is sparse, contradictory and based on erroneous assumptions, making extremely difficult the interpretation of data. This is the case of paracetamol, as shown by Nunes et al. [1]. According to this study, the toxicity of paracetamol is highly variable, even among species of the same phylogeny; however, this situation is even more complicated if one considers the variations of magnitude in responses obtained with standardized bioassays.

More than being an exhaustive attempt to establish a comprehensive review of what has been done in recent years regarding the diagnostic of effects of drugs in the wild, the present chapter intends to summarize the new evidences showing that therapeutic drugs and their residues/metabolites can indeed work as environmental pollutants, and may constitute additional sources of chemical stress to already polluted areas. It is also our intention to show the linkage between exposures to low, almost vestigial, levels of pollutants, and the most significant biological deleterious effects, in several biological models, mainly from the aquatic environ-

ment. Both field and laboratory studies will serve as case studies of particular importance to demonstrate that pharmaceuticals, despite their almost negligible concentrations, can be of environmental concern for sensitive key elements of the ecosystem. On the other hand, one of the main purposes of this review is the establishment of key guidelines, for the development, implementation and validation of toxicological biomarker tools to assess the subtle effects elicited by pharmaceuticals.

The issue of pharmaceuticals as contaminants has been a hot topic in environmental sciences for more than two decades [2]. However, this is not a novel issue, and early studies conducted during the mid 70s already showed the presence of significant amounts of clofibric acid (the pharmacologically active metabolite of several fibrates that explains their activity as lipid lowering agents), in water from the sewage systems of a North American town, Kansas City [3]. This same compound was again found in water quality monitoring studies, initially aimed to quantify pesticide residues, in Germany [4], almost twenty years after their initial detection. This apparent coincidence meant that compounds such as clofibric acid might have a general and ubiquitous presence, being highly dispersed among water compartments [5]. This was then confirmed by subsequent studies, showing that the dispersion of these pharmaceutical substances was not limited in any way to sewage or even freshwater, since it could also be detected in the North Sea. Clofibric acid has an undisputable historical importance, that was not followed by the confirmation of its (eco)toxicological significance [7, 8]; nevertheless, and from a merely retrospective analysis, its detection in several water samples was a major event that served as basis for a new area of environmental toxicology, devoted to the study of the presence and effects of therapeutic drugs in the environment.

Given the enormous body of evidence that was compiled since the mid 90s to the present day, from studies involving all possible aquatic matrices (freshwater, sea water, sewage effluents, drinking water, groundwater) it is almost impossible not to consider the issue of drugs and their ecotoxicological effects one of the most challenging scenarios for years to come. Consequently, the presence of pharmaceutical residues in the wild is nowadays a matter of interest, among the scientific community and the general public [1, 9, 10, 11]. This interest derives from the intrinsic features that these compound possess. Pharmaceuticals are biologically active, capable of exerting effects in a large number of organisms, even when in extremely low concentrations. Drugs are widely used and dispersed, being ubiquitously found in the aquatic environment, as a result of the overall low degradation efficiency of sewage treatment plants; drugs are present in surface water, groundwater, and even oceans; furthermore, these substances are refractory to biological degradation or can assume other forms after metabolism [2, 9, 12], largely toxicologically uncharacterised especially for wild organisms. An adequate lipophylicity allows drugs to be slightly water soluble, but readily absorbed by living organisms [8, 13]. Aquatic organisms are by far more exposed to pharmaceutical residues. The deleterious impact of specific therapeutic compounds on aquatic organisms has already been shown to occur, even under real scenarios of contamination [13, 14, 15].

Drugs reach the aquatic compartment mainly via sewage systems. The use of pharmaceutical drugs requires its ultimate elimination from the patients' organisms, which results in its presence in the sewage treatment system, when it is present [2]. In modern western societies, sewage treatment plants (STPs) are common and generally efficient. However, the purpose of

conventional STPs is reducing the amount of organic pollution, and not the elimination of often-recalcitrant compounds such as drugs. This results frequently in extremely low removal rates in STPs when it concerns to pharmaceuticals [16], requiring the implementation of novel and usually expensive technologies. This results in the continuous release of drugs and their metabolites into the receiving waters. Given that the amount introduced into the wild generally equals the sum of drug that is naturally degraded by natural pathways, it is possible to sustain that pharmaceuticals are environmentally pseudopersistent [17, 18]. Other alternative routes can also explain the presence of pharmaceuticals in the aquatic compartment, but in a lesser extent, such as release from manufacturing industries, and leachates from landfills [19]. Despite the existence of distinct routes by which pharmaceutical substances reach water bodies, it is important to stress that the majority of the residues result from human use and release, and from the inefficacy of treatments systems. Consequently, the issue of aquatic contamination by drugs is intrinsically connected to the personal use made by human consumers, which cannot be stopped or prevented, even if more advanced solutions to mitigate the presence of drugs are developed and implemented.

As a consequence of human use, several classes of drugs are routinely detected and quantified in the most varied water matrices. The most prominent classes of drugs found in the wild include non steroidal anti-inflammatories, antibiotics, anticonvulsants, antidepressants and oral contraceptives, which are systematically reported in monitoring surveys [20, 21] of the aquatic environment. However, this corresponds to a generic assumption and the reality shows that almost all substances (or their metabolites/degradation products) used in human therapeutics can be virtually detected, mainly in sewage or even in receiving waters. It is also noteworthy to observe that representatives of all these pharmacotherapeutic classes co-occur, simultaneously, in the same sample or matrix. Despite co-occurring in extremely low amounts, usually ranging from the ng to the  $\mu\text{L}$  per litre, it is not possible to discard the possibility of exertion of effects, mediated or not by the same receptors activated during human therapy. This poses important challenges, not only in analytical terms (which are out of the scope of this chapter), but especially in terms of the toxicological deleterious outcomes resulting from exposure to such complex mixtures, in individual terms (altered physiology of exposed organisms) and to the ecosystem [22]. Given these main topics, the major scientific question addressed here can be described as an interconnected two-tier approach: do pharmaceutical drugs, or metabolites/residues, exert deleterious effects in wildlife? If so, what is the type of effects to be expected, and what the extent to be considered? To answer these two issues, it will be necessary to adopt new strategies to surpass the usual difficulties in obtaining responses or measurable biological effects. Until the present day, few studies clearly showed the relationship between realistic conditions of exposure and deleterious effects caused by pharmaceuticals in non-target organisms. Considering the most frequently adopted toxicological endpoints (e.g. death, growth impairment) and the levels, concentrations or dosages required to elicit such effects, it is possible to state that traditional approaches are not suited, for most cases, to address the effects of drugs in aquatic organisms. Thus, it is mandatory to select an additional set of tools that may address the issues initially raised, and constitute future testing guidelines for pharmaceuticals in the wild. The combination of standardized methods, well-established analytical techniques, and new biochemical strategies (including gene



expression/epigenetics) might result in the establishment of a link between the low levels of exposure and biological responses in non target, environmentally exposed biota.

## **2. Effects to be expected from drug exposure**

It is impossible to predict the effects of drugs in the wild, given their sheer number and the possible interactions among them in the wild. The mere quantity of different drugs in use in modern human therapeutics is overwhelming, and prevents the establishment of any plausible prediction in terms of toxicity of complex mixtures, such as urban effluents. The human use of pharmaceutical drugs in the European Union is vast, and approximately 3000 distinct substances are used, including substances from different pharmaco-therapeutic classes such as anti-inflammatories,  $\beta$ -blockers, oral contraceptives, blood lipid regulators, antibiotics and others [23]. This is a brief, albeit comprehensive summary of the therapeutic classes one can find in the aquatic compartment, from an empirical perspective of only considering classes of drugs that are used in extremely high amounts. However, this is a criterion that is not exempt of drawbacks or criticisms, since the mentioned classes, those that are used and dispersed in the highest amounts, are not necessarily representative of drugs with the highest biological or toxicological activity. For instance, cytostatic or anticancer drugs are extremely active and biologically aggressive, despite not being extensively used [24]; however, these substances are among the most active environmental drugs [25]. It is thus extremely difficult to prioritise substances only based on simple criteria of use and consumption; more difficult is the task to develop and validate markers of toxicological interest to be used in routine analysis.

Furthermore, the presence of an even larger number of metabolites [26], and products of degradation by natural or anthropogenic means (photodegradation, hydrolysis, microbial degradation, chemical treatment processes at STPs, chemical reaction among drug residues and with other substances) implies the need to include the possibility of toxicological interactions among all compounds that may be present in a given environmental (especially water) sample; these interactions, that may result in increased toxicological activity, has been already shown to be a possibility in the wild [27, 28]. It is not just a matter of selecting a biological response, but to choose the one most likely to respond to this vast group of compounds, in a specific organism that may be successfully analysed, both in field surveys and in laboratory-based bioassays. It is not just feasible to study all pharmaceutical compounds, on all putative model organisms.

## **3. Typology of toxic effects elicited by drugs and its relation with pharmacology**

The toxicity of pharmaceutical drugs in exposed aquatic biota is frequently determined by their intrinsic pharmacology and toxicology, and outcomes that are already described for other species are also possible to occur in aquatic organisms [29]. Additionally, other toxic effects may not derive at all from the known pharmacology of these substances, and can indeed result

from specific biochemical and physiological pathways that are over-stimulated in highly responsive species. Thus, it is possible to expect a wide range of toxic effects, which difficult the process of selection of adequate and responsive toxicological endpoints to be observed and studied, both in monitoring and in laboratory based assays.

Despite the multiplicity of effects drugs can cause on non-target aquatic organisms, the selection of a marker of toxicity is sometimes extremely difficult. It is impossible to select a single biomarker that will be equally responsive to all drugs, considering the diversity of pharmacological mechanisms involved in the activities of such a large number of substances. Consequently, it must be emphasized that a thorough process of selection of adequate markers of toxicity is mandatory. However, the quest for a putative toxic effect of a drug can be directly connected to its mode of action, in pure pharmacological terms. This was the case of anticholinesterasic compounds of therapeutic use, such as pyridostigmine and neostigmine, as shown by Rocha et al. [30]. This study showed that the most likely effects of environmental contamination by these two substances could be related to cholinesterasic impairment, a key factor encompassing other effects at the individual and population level, such as reduced feeding behaviour and decrease offspring production. However, this study was not followed by other successful attempts to establish relationships between drug exposure and potential ecotoxicological effects, and very scarce is the number of published papers that point this possibility. The study conducted by Rodrigues et al. [15] evidenced that the same drug, pyridostigmine, could elicit similar results in the fish species *Lepomis gibbosus*, in terms of cholinesterasic inhibition; however, this pattern of response was not reflected by behavioural modifications, despite the occurrence of neurotoxicity. Drugs can act by pathways that are also shared by other classes of compounds, which share with drugs the same aquatic matrix. Cholinesterasic inhibition was again the target of the study by Nunes et al. [31], when studying the combination effects of a pharmaceutical drug, pyridostigmine, and two common environmental contaminants (the metal copper, and the organophosphate pesticide chlorfenvinphos), well known for their ability to impair cholinesterasic activity of exposed aquatic organisms. This study showed that the combination of the three compounds, even for realistic levels, could result in a toxicological outcome that constitutes the exacerbation of the pharmacological pathway activated by the drug pyridostigmine.

The environmental effects of other substances of therapeutic use are also related with their intrinsic effects. One of the most thoroughly characterized examples is the one of antibiotics. By being discharged into the aquatic environment, often maintaining intact their pharmacological properties, antibiotics still exert their effects on wild bacterial species [32]. This favours the selection of resistant strains, by means of dispersing resistance genes among susceptible bacteria. This phenomenon has been extensively characterized, and derives from the anthropogenic pressure exerted by human residues containing drugs that are released into the environment, favouring the dispersion of ancient natural genes that encode resistance mechanisms [33, 34]. To address the issue of antibiotic presence and effects in the environment, namely those that can increase gene transfer among bacterial strains, a common strategy is to analyse where these genes can be found, especially in the water compartment, which is the most vulnerable to this issue. The review by Zhang et al. [35] summarizes the efforts recently devoted to the development of new methodologies to characterize the dissemination of resistance genes in the wild following antibiotic release from human activities. It is thus

possible to conclude that the mere analysis of genes encoding resistance factors can be considered an effective tool to analyse the effects of antibiotics in the wild.

Effects of other drugs can involve alterations in the regulation of the endocrine system. The use of synthetic oral contraceptives, glucocorticoids and others is one of the main causes for the ubiquitous presence of these classes in most aquatic matrices, where they maintain their biological effects [36]. Some of these compounds have already been demonstrated to exert potential endocrine effects in exposed wildlife, even in the ranges of concentrations in which they were found [37]. Despite their present levels of contamination, which are indisputably low, the large use and liberation of these compounds into the aquatic compartment will not exempt this endocrine disrupting substances from exerting deleterious effects on exposed biota, as reviewed by Runnalls et al. [38]. In fact, the effects caused by this class of compounds have already been documented, especially in terms of development, metamorphosis, and sexual dimorphism, in amphibians [39], fish [40, 41], molluscs [42], and also crustaceans [43]. It is not surprising that these compounds, even after excretion, maintain their initial pharmacological properties. However, effects elicited are well distinct from those caused in humans, and can include feminization and significant alterations in individual features and population structures, which constitutes a major effect in ecological terms. So, it is possible to conclude that the assessment of endocrine disrupting effects is another valid example on how to use the pharmacological properties of a given class of chemical drugs to search for their effects in the environment. The study conducted by Velasco-Santamaría et al. [44] evidenced that the presence of such compounds in the wild can also be potentiated by the concomitant presence of other substances with similar pharmacological properties and therapeutic use. The synthetic oestrogen ethinylestradiol can exert increased toxic endocrine effects in the presence of another endocrine active pharmaceutical, such as trenbolone, in the marine fish species *Zoarces viviparus*.

#### 4. Mortality, growth impairment and other classic tools

Despite their traditional use for the toxicity assessment of a large number of substances, mortality and growth impairment endpoints have been extensively used to describe the toxicity of pharmaceutical substances towards wild biota. In fact, more than reflecting real scenarios of contamination, the validity of calculating and interpreting mortality data relies on the establishment of sublethal toxicity criteria and to analyse in comparative terms, the ecotoxicity of substances that act by unknown (or largely uncharacterised) mechanisms of toxic action. In fact,  $LC_{50}$  values of pharmaceutical drugs are well suited to establish rankings of ecotoxicity among very distinct compounds, and these criteria are straightforward to implement and interpret. However, these endpoints are not a first choice if one requires the establishment of complex mechanisms, or even effects at extremely low levels, which are likely to cause alterations other than death, immobilization, or impairment of the population growth. These responses are not fine tuned alterations, and may be seen as blunt tools for toxicity characterization, with poor or even null ecological relevance.

The number of ecotoxicity studies analysing lethality or growth impairment is thus considerable. The study published by Carlsson et al. [45], showed that antiparasitic drugs could exert



extreme toxicity, when compared to antibiotics, thus threatening the survival of zebrafish embryos. The crustacean species *Daphnia magna* was very sensitive to the antidepressant sertraline, as shown by Minagh et al. [46]; this compound was capable of inducing strong alterations in the population of this crustacean species, after 21 days of exposure. Population changes were also observed after chronically exposing *D. magna* to the compounds testosterone and 4-hydroxyandrostenedione, even for ecologically relevant levels [47], reinforcing the possibility of exertion of deleterious effects in the wild. Another crustacean, namely *Neocaridina denticulate*, demonstrated to be extremely sensitive in terms of lethality to a combination of pharmaceutical drugs (paracetamol and ibuprofen) commonly found in the aquatic environment [48]. The ecotoxicological effects of mefenamic acid on *D. magna* and *Moina macrocopa* were evaluated by Collard et al. [49]. This study showed that lethal effects were not likely to occur for already reported levels of this compound; however, the chronic exposure to this compound showed that changes in reproduction are possible, especially in the case of *M. macrocopa*.

On the other hand, the absence of potential risk posed by the presence of clofibric acid was evidenced by the study by Nunes et al. [50] and by Emblidge and DeLorenzo [51], in multi-species assessments that focused on mortality and growth impairment as toxicity endpoints. Similarly, the work by Ferreira et al. [52] evidenced the absence of potential lethal effects posed by antibiotics (oxytetracycline and florfenicol) on the crustacean species *Artemia parthenogenetica*. Despite not exerting lethal effects, several combinations of pharmaceutical drugs (including an ecologically relevant mixture) were capable of causing significant alterations in zebrafish embryos, as shown by Madureira et al. [53]. The antibiotic oxytetracycline was shown to be reasonably safe towards the fish species *Labeo rohita*, since the lethal levels were well above concentrations that are not likely to be found in the wild, as shown by Ambili et al. [54].

Growth of autotrophic organisms is another endpoint likely to respond to exposure to human use drugs; the study conducted by Berninger et al. [55] showed the refractivity of the aquatic plant species *Lemna minor* to the drug diphenhydramine. Somewhat similarly, the work conducted by Nunes et al. [56] evidenced the occurrence of an oxidative-based response of two species of the genus *Lemna* (*L. minor* and *L. gibba*) elicited by the drug paracetamol, which resulted in significant growth alterations. On the other hand, the study conducted by Ferreira et al. [52] presented evidences concerning the toxicity of the antibiotics oxytetracycline and florfenicol on the microalgae species *Tetraselmis chuii*.

## 5. The quest for new biomarker tools: Oxidative stress

As seen before, the transformation of a pharmacological effect into a biomarker, can thus work as a reliable indicator of exposure of wild organisms to several classes of pharmaceutical drugs. Despite the presented evidences, describing successful cases in which the pharmacology of a drug (or a group of drugs) may also be used to study its toxicity, the majority of therapeutic drugs do not exert effects that can be interpreted as reliable environmental biomarkers. There are two major drawbacks of this approach: the first reason is related with the levels in which these substances are found, and the second most important, is the absence of a counterpart response in wild organisms of the response elicited in human patients.

Toxicity is a matter of dose, and can be generally described by a dose-response relationship. Assuming that these compounds are found in extremely low levels, the exertion of a clear toxic response (e.g. death, immobilization, impairment of reproduction) is not always possible. It is thus important to know in detail the mechanisms by which the drugs not only exert their pharmacological activities, but also those involved in adverse effects, metabolism and detoxification processes, and other accessory or side-effects that the drugs may cause. Only by means of knowing these fundamentals of pharmacology and toxicology it is possible to select biomarkers that will respond satisfactorily at extremely low levels of exposure. Given the extremely low levels in which drugs are found in the aquatic compartment, effects are sometimes minor and negligible, or may even be considered null or absent if an adequately responsive biomarker is not employed. This can be a real challenge for ecotoxicologists, since specific biomarkers, others than the main pharmacological effect, that signal a biological alteration following an exposure to drugs in concentrations between the ng/l to µg/l are not abundant.

On the other hand, drugs are designed to be safe for human patients, and it is possible to suggest that this somewhat harmless nature can also prevent drugs from causing extreme toxicity in other organisms. However, drugs can indeed cause multiple effects, which do not occur by activation of specific single receptors, but reflect major changes in the homeostasis of exposed organisms. The presence of pharmaceutical compounds can cause changes at many different levels, including in the redox cycle of exposed organisms; these may cause the formation of reactive chemical species, capable of inducing damages to biological structures. Therefore, the quantification of oxidative stress biomarkers is important to evaluate the redox status of the exposed organisms. Several studies have already shown that, even for realistic levels of contamination, some compounds can exert pro-oxidative effects, measurable mostly in terms of modification of the antioxidant defence mechanism of exposed aquatic organisms; in many cases, membrane lipid peroxidation is a likely outcome of oxidative damage, and this event can be also quantified [8, 13, 56, 57, 58, 59]. The exertion of oxidative stress is a factor to consider when evaluating long term, thus realistic, exposures. Considering that exposure to anthropogenic therapeutic drugs can occur during a significant portion of the entire life cycle of a given organism, it is not possible to exclude the occurrence of cumulative processes ending up in irreversible conditions. In fact, oxidative stress, if sustained for long period, can also be the cause of genotoxicity. Data from the literature sustain the exertion of deleterious effects of specific pharmaceutical compounds, including genotoxicity on fish [60], and in crustaceans (e.g. *D. magna*) [61, 62]. Consequently, it is possible to use oxidative stress not only as an indicator of exposure to a broad series of compounds, but also as a predictor of other subsequent effects, that may derive from oxidative alterations and damages.

The extended knowledge concerning the metabolic pathways required for the bioactivation or detoxification of therapeutic compounds can also function as a valid source of analytic tools for the assessment of effects. The activation by over-expression of metabolic enzymes, involved in the detoxification of pharmaceuticals, is a probable event whenever a biological system is challenged by exposure to drugs in the wild. Among metabolic enzymes prone to over-expression, one can identify mainly those involved in the direct metabolism of drugs. The activities of phase I metabolic enzymes, for example (such as cytochrome P450), and phase II metabolic reactions (namely isoenzymes glutathione-S-transferases, GSTs), can also be

increased in aquatic organisms by exposure to pharmaceutical drugs [7, 8, 13]. Being routinely quantified in modern ecotoxicological laboratories, the activities of such enzymes are intrinsically interesting to quantify not only the level of exposure to drugs, but also the likely consequences of drug exposure. This may be justified since these enzymatic forms are not only involved in the bioactivation/detoxification of drugs, but do also participate in numerous endogenous processes, which can be altered following an environmentally drug-induced chemical insult. The broad spectrum of these analytical tools is also a factor to consider, being highly unspecific, effects both phase I and II metabolic enzymes can indeed respond to a multiplicity of therapeutic classes.

## 6. Alternative tools to assess the environmental effects of drugs: Toxicity, pharmacology and other effects

Several toxicity assessment projects have relied in the development and validation of new tools to quantify the extent of the toxic response. As previously stated, known pharmacological properties can serve as a comprehensive source of biomarkers to be used in ecotoxicity assessments. However, some of the responses of wild organisms to drugs may be based on physiological mechanisms that are not directly related (activated or impaired) following patterns included in the pharmacology of pharmaceutical substances. Some of these responses are purely paradoxical, while others are only the reflex of the activation of mechanisms and receptors in wild organisms that were never studied and/or identified in common experimental models.

This is the case of behavioural alterations in several wild organisms. The work by Berninger et al. [55] showed that the fish species *Pimephales promelas* was highly sensitive to the antihistaminic drug diphenhydramine in terms of feeding behaviour. The feeding behaviour was also modulated after exposure of the fish *Perca fluviatilis* to the antidepressant sertraline, as evidenced by Hedgespeth et al. [63]. Behaviour is also a trait that can be significantly changed after exposure to pharmaceuticals, both in fish [64], and in crustacean species [65]. Strong behavioural alterations were also reported by Nunes et al. [13] after the exposure of the fish *Gambusia holbrooki* to the neuroactive compound diazepam, with impairment of the swimming capability. An opposite pattern has been presented by crustacean species, which seem not to be equally responsive to pharmaceutical drugs. As evidenced by Nieto et al [66], the food ingestion behaviour of the freshwater crustacean *Atyaephyra desmarestii* was not affected by ecologically relevant levels of several therapeutic drugs, such as diclofenac, ibuprofen, and carbamazepine. Food ingestion was also affected following exposure of *Xenopus laevis* to fluoxetine, as demonstrated by Connors et al. [67], thus conditioning the development of this organism during its early life stages. Antidepressants that exert their therapeutic activity through the selective inhibition of serotonin reuptake are likely to be adequate candidates to alter the behaviour of a large number of aquatic organisms, considering that the most prominent pathway involved in their activity is highly conserved. According to the editorial by Ford [68], specific compounds including sertraline and fluoxetine, can dramatically alter the feeding behaviour profile of a large number of aquatic organisms, from fish to crustaceans.

The endocrine disrupting effects of several human use drugs has been the subject of research for several years, and quite a few studies report the occurrence of significant effects caused by drugs (e.g. anti-inflammatories) on fish [69]. Endocrine disruptive effects caused by pharmaceuticals are not exclusive to fish, since invertebrates, such as crustaceans, are also prone to be affected in their endocrine functions by exposure to pharmaceutical drugs, as reviewed by Hutchinson [70]. Neuroendocrine effects are another aspect of this issue. Considering that a large number of pharmaceutical drugs act by altering the expression and effects of biological compounds of high physiological importance (e.g. neuropeptides, neurotransmitters, or neurohormones), it is with no surprise that similar mechanisms can be impaired in non-target species environmentally exposed to these same drugs. The consequences are not only so far uncharacterised, but also, unpredictable. Consequences to be expected will naturally include alterations in the physiology of exposed wildlife, affecting behavioural traits, or the hormonal homeostasis, which are of fundamental importance to the organisms and to the ecosystem. It is thus expectable to observe impairments at several levels, such as reproduction, development, growth, response to chemical aggression or other sources of stress [71, 72]. The neuroendocrine effects of specific compounds, such as sertraline, were shown by Connors et al. [67] in tadpoles of the species *Xenopus laevis*. This antidepressant substance caused significant developmental impairments during the early life stages of this organism, which occurred for ecologically relevant levels. Another antidepressant drug, such as fluoxetine, was also capable of inducing strong alterations in the reproductive physiology of the fish species, *Carassius auratus*. Another antidepressant, mianserin, was also related to estrogenic activity in fish (*Danio rerio*) by inducing molecular biomarkers of estrogenicity (such as vitellogenin1 and zona pellucida proteins), as evidenced by van der Ven et al. [73]. The study conducted by Mennigen et al. [74] concluded that exposure to relevant levels of this substance could alter the expression and release of several physiological hormones, thus compromising the sexual behaviour of this fish species. Therapeutic drugs such as paracetamol and lincomycin are also involved in endocrine disruption effects. The study conducted by Kim et al. [75] showed that these two pharmaceuticals could affect the steroidogenic pathway and increase estrogenicity, in crustaceans (*D. magna* and *Moina macrocopa*), but also in fish (*Oryzias latipes*). These effects were translated into a significant reduction in juvenile survival of fish, and on a significant increase in the vitellogenin levels in male fish. Other substances, such as furosemide and several fibrates (e.g. bezafibrate, fenofibrate and gemfibrozil) can also exert this type of endocrine effects. According to the data obtained by Isidori et al. [76], these substances were shown to activate the human estrogenic receptor  $\alpha$ , thus favouring estrogenic responses in wild organisms. Mefenamic acid is another example of an endocrine compound whose pharmacology in most experimental organisms does not include this aspect. However, the data compiled by Collard et al. [49] showed its involvement in endocrine effects in fish (*D. rerio*), evidenced by alterations in vitellogenin and its mRNA expression, overexpression of genes of the hypothalamus–pituitary–gonad axis, and histological changes in ovaries of exposed females.

Epigenetic effects can also derive from the environmentally-driven impact of specific compounds; exposure to persistent organic pollutants (including pharmaceuticals) or endocrine disrupting chemicals are examples of classes of chemicals that have been related to alterations in epigenetic marks, including in fish and cladocerans (Vandeghechuchte and Janssen, 2011) [77].



Several published papers refer that deleterious effects of transient chemical exposure (namely, via environment) of *D. magna* can result in the transference to nonexposed generations through epigenetic inheritance [78, 79, 80], which is a decisive factor to link ecotoxicological effects observed at the levels of communities to alterations at the ecosystem levels [81]. The effects of chemical pollutants on the epigenetics of fish is also significant, as shown by the screening of pollution resistance of north American fish species [82]. Alteration of gene expression is also another factor to consider after environmental exposure to chemical stressors; several papers show the responsiveness of aquatic organisms to environmental pharmaceuticals, demonstrating the validity of this approach [83, 84].

Specific drugs, not anticholinesterasic by nature, can also impair neurotransmission, by cholinesterasic inhibition [85]. One of the most significant examples is the one represented by zinc pyrithione. According to the work developed by Sánchez-Bayoa and Goka [86], this anti-dandruff compound is extremely toxic to several aquatic organisms, including the crustacean *D. magna*. Despite being photodegradable, recent studies show that zinc pyrithione may exert important toxic effects on aquatic organisms (e.g. *Paracentrotus lividus* and *Mytilus edulis*), even at extremely low levels [87]. Effects of zinc pyrithione are not restricted to invertebrates, since fish species are also extremely sensitive to the presence of this compound [88]. The products of degradation of zinc pyrithione can be of great environmental concern per se, since the effects of such compounds on several marine organisms are well known. The toxicity of zinc pyrithione has been documented for organisms such as the algae species *Skeletonema costatum*, the crustacean *Tigriopus japonicus*, and the fish *Pagrus major* [89]. The mechanism of toxic action of zinc pyrithione metabolites includes AChE inhibition, as shown by Mochida et al. [90].

The energy metabolism of wild organisms is a putative target for pharmaceutical toxicity. As shown by Mennigen et al. [91], exposure to the drug fluoxetine could result in significant alterations in the fish species *Carassius auratus*, namely in terms of energy metabolism. Low levels of exposure were causative of anorectic effects, while higher levels could directly compromise the hepatic glucose metabolism, by means of depressing the activity of the gluconeogenic enzyme fructose-1,6-bisphosphatase. Chronic exposure of marine mussels (*Mytilus* sp.) to two therapeutic drugs, genfibrozil and diclofenac, showed the interference of these substances on several parameters, including energy metabolism features [92]. The respiratory activity of exposed organisms is another function that can be altered after exposure to anthropogenic compounds, which interfere with metabolic pathways used by organisms to obtain energy (anaerobiosis vs. aerobiosis) [7]. This study evidenced the roles of both clofibrate and clofibric acid, hypolipidemic fibrates used in human therapeutics, in the increase in muscle lactate dehydrogenase activity, thus favouring the less energetically efficient anaerobic pathway.

## 7. Future directions: A combination of tools

From the previous sections, it was made clear that it is extremely difficult to search and define without any shadow of doubt the biological effects to be expected from a large number of



extremely different chemical substances, exerted on a vast multiplicity of organisms. Not only are the substances very different per se, thus exerting distinct effects, but also the organisms can have alternative pathways and receptors that make them more or less prone to the exertion of those same effects. It is not possible always to rely on the well-described human pharmacology, despite the large number of studies that sustain the most common effects, since humans are not environmentally exposed to the majority of these substances or to their residues. However, some of the effects are shared both by humans and by other organisms in the wild, a decisive factor when one tries to select an adequate tool to quantify an effect elicited by a pharmaceutical drug in the environment.

The present scenario shows us that we are halfway between the total lack of data concerning pharmaceutical effects in the wild, and a full and comprehensive knowledge about their faith and ultimate consequences. A large effort has been undertaken and toxicity of several pharmaceutical classes is nowadays already characterized in a vast number of organisms. Despite the validity of this effort, other pharmaceutical classes are not fully understood in their interaction with biota, thus requiring the development of additional attempts until a definite light is shed on this issue. Presently, environmental scientists dealing with this issue still have to face a significant array of drawbacks, from the simple lack of data for some drugs/organisms, unpredictability of data and, of the biological responses in somewhat exotic species, confounding factors that already occur for other studies, but whose influence is exponentially increased in this specific area, lack of analytical tools, such as biomarkers with enough sensitivity to face the extremely low levels found in the wild and that are able to understand highly subtle biological responses, lack of test protocols or species well adapted to be used in ecotoxicological testing under conditions of brackish water, tropical or arctic climates, or extreme environments, in which drugs are also likely to occur.

Despite not being the core of this chapter, the quantification of the levels of pharmaceutical drugs in the wild, especially in the aquatic environment, is crucial. It is important to know in detail the compounds that may exist (or co-exist) in the same matrix, since these compounds are important to select an analytical tool/biomarker that will allow the prediction of biological effects. It is also of fundamental importance to know which are the most representative compounds (or pharmacotherapeutic classes) in a given sample also to establish causal relationships between their levels and the extent of the observed biological response. Only with a complimentary approach comprising hydrology, water analytical chemistry and biological assessment of effects it will be possible to fully characterize the impact of drugs on the wildlife.

The typology of exposure is also a matter of concern. From the previously mentioned studies, two main types of exposure were adopted in the majority of studies: acute (short-term) and chronic (long term). The use of short-term exposure periods is somewhat neglected, but this is not a totally invalid strategy. Despite not having the relevance of a long period of exposure, which reflects the most likely conditions of exposure in the wild, short exposure periods are also of extreme importance, and must be included in bioassays for the assessment of the effects of pharmaceutical drug. Acute assays are important since they allow researchers to test the responsiveness of a specific test species towards a given drugs, Acute exposure can also be of

importance to define rankings of comparative toxicity for several substances, independently from their mode of action and toxicity. Data from acute tests can also be useful to determine ranges of concentrations representing sublethal levels to which organisms may be exposed. Finally, the entire set of information potentially gathered from this type of test may serve to prioritise compounds to be studied under chronic conditions of exposure. However, and if one considers the need to increase the ecological relevance of data obtained from ecotoxicity tests, chronic assays are likely to represent a more credible simulation of what happens in the wild. Organisms are frequently subjected to contamination during considerable periods of their entire life cycle, or may even contact with chemical pollution of anthropogenic origin for different generations. It is thus important to prioritise a testing strategy that simulates these conditions, and the most adopted type of bioassay, despite its inherent difficulties, is the chronic exposure. Chronic exposures can more easily mimic real events, occurring under realistic low levels of contaminants, and can consequently increase the ecological relevance of the obtained data. Furthermore, the selection of chronic exposures can permit the proposal of multispecies assessments (e.g. mesocosms), which are obviously advantageous if one intends to simulate real environmental conditions. On the other hand, multispecies assessments are a valid approach, since the sensitivity of distinct organisms towards pharmaceuticals is frequently very diverse.

From the majority of the cited studies, it is possible to conclude that a biomarker-based approach is valid to obtain information regarding specific pathways involved in the toxic response. This does not necessarily imply that more traditional approaches (including mortality, or growth/population effects) are fully inadequate to assess the ecotoxicological effects of drugs. Nevertheless, the low levels of exposure make difficult the exertion of such effects, and the resultant toxicity often occurs by impairment of specific, subtler, biochemical pathways. It is thus important to analyse the sub-individual level, and more biomarkers must be proposed and fully validated. Effects at the molecular level, including enzymes, must be interpreted as signalling tools for effect or damage in biological systems. Given their overall importance, specific pathways must be primary sources of analytical tools. It is possible to suggest that novel biomarkers can derive from analysis of the enzymatic machinery involved in the energetic metabolism, gene expression and epigenetics, and damage (e.g. of oxidative nature) repair. These will be the most likely biomarkers of contamination of the future.

The next step will be transposing laboratory biomarker-based assays, with a combination of chronic-acute exposure of multiple species to other alternative models, namely under field conditions. It is now mandatory to propose new test species, well adapted to conditions that do not represent standard settings: species from tropical/polar (or otherwise extreme) regions must be analysed following the above-described strategy, and their use for ecotoxicological purposes validated. This will be of crucial importance to transfer bioassays from the laboratory to the field, increasing the validity of data and of the conclusions drawn.

Finally, a last step will combine the simultaneous analysis of complex mixtures of drugs. Frequently, environmental matrices are contaminated by a large number of distinct pharmaceuticals; any analytical procedure based on the quantification of effects caused by a single chemical will always be unsatisfactory, and will underestimate the actual toxicity of complex

but realistic mixtures. To avoid this underestimation, it will be of the uttermost importance to know the general interaction profiles that may occur for a large number of test species, caused not by isolated single compounds, but by the main representatives of known therapeutic classes: not being possible to test the potential interactions occurring by the simultaneous presence of hundreds or even thousands of compounds in the same matrix, a more systematic approach may involve the definition and characterization of putative biological relations of pharmacotherapeutic classes among each other.

## 8. The effects of drugs in the wild

Despite the considerable number of cited research articles so far, the present chapter would not be satisfactorily summarized without a critical evaluation of the potential ecological damages posed by pharmaceutical residues. The major drawbacks for the analysis of effects caused by pharmaceuticals are also the most important defence against their risks: their extremely low levels. Being present in residual amounts, sometimes below the ng/l range, the majority of drugs do not attain levels capable of exerting effects. However, the simultaneous presence of compounds that act via a similar pathway may favour the exertion of effects. Still, few are the examples of substances for which this behaviour has been described. Despite the lack of reported effects, and the impossibility so far of establishing a direct, unequivocal relationship between pharmaceutical exposure and deleterious effects in wildlife, an increasing number of studies has brought the issue of ecological relevance of data to the discussion. By testing already reported levels of contaminants, some researchers have already claimed having demonstrated the putative effects of drugs in exposed organisms that are also likely to occur in the wild. This has been the case of several fibrates [7], synthetic hormones [93], ivermectin [94], paracetamol [1, 57, 59], ibuprofen [95], neostigmine and pyridostigmine [15, 30, 31], fluoxetine and other antidepressants [93, 96]. However, the majority of studies published so far do not demonstrate this intrinsic association between pharmaceuticals contamination and deleterious effects. Nevertheless, research studies on this matter have clearly demonstrated the responsiveness of a large number of species towards drugs, evidencing the potential for toxic effects if a threshold level is attained and surpassed. This is of the uttermost importance, since it clearly shows that some of the highly conserved pathways used and activated by pharmaceutical drugs in humans, are also present in a significant number of wild organisms; this increases the possibility of biological-chemical interaction, with sometimes totally unpredicted overall effects. The protection created against the effects of human drugs by their low levels can thus be simply temporary, considering the ever-increasing amount of drugs and their residues in the wild.

## 9. Conclusions

The need to understand the potential effects of a large number of biologically active substances in the wild, has driven researchers to the development of new assessment methodologies.

Unlike other substances with human origin, pharmaceutical drugs are active, and may pose significant risks even after their elimination and for long periods. Being excreted in extremely high amounts and on a daily basis, such substances are pseudopersistent and recalcitrant. Not even the implementation of dedicated sewage treatment systems endured to reduce the global amounts of drugs entering especially the aquatic environment. Consequently, the presence of such substances is now a global reality, needing to be dully characterized. Non-target species are the most likely and vulnerable targets for the exertion of deleterious effects. The study of putative toxic effects requires the development, implementation and validation of novel analytic tools, specifically devoted to the particularities of drug contamination. From the revised literature, it is possible to anticipate that ecotoxicological analysis, in the future, will require the combination of distinct tools, on a complementary basis. The tools to be used in the future will not only respond to extremely low levels of contamination, but will include signalling responses well suited to diagnose exposure to specific classes of drugs. Despite not being entirely adapted to the issue of contamination by low levels of pharmaceutical drugs, standardized bioassays can be a valuable tool, if complemented with adequate molecular and subindividual endpoints. Being impossible to characterize the entire set of toxic responses elicited by single, individual compounds, it will be important to know the most important toxic responses of common pharmacotherapeutic classes, to allow the prediction of potential interactions. The use of multispecies assessments will also be important, since the sensitivity towards specific compounds is not necessarily comparable among distinct test organisms. Long-term studies, favouring phenomena of bioaccumulation during important periods of the organisms' life cycles will allow knowing in detail the potential endocrine effects elicited by specific compounds. Behaviour is another feature likely to be altered after pharmaceuticals exposure, thus requiring the development of new testing methodologies to address this issue.

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## Author details

Bruno Nunes\*

Address all correspondence to: nunes.b@ua.pt

Departamento de Biologia, Centro de Estudos Ambientais e do Mar (CESAM), Universidade de Aveiro, Campus Universitário de Santiago, Aveiro, Portugal

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